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Original article

Iron regulated genes of *Salmonella enterica* serovar Typhimurium in response to norepinephrine and the requirement of *fepDGC* for norepinephrine-enhanced growth[☆]

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Abstract

Catecholamines may stimulate enteric bacteria including the foodborne pathogen *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) by two mechanisms in vivo: as a quorum sensing signal and a supplier of iron. To identify genes of *Salmonella* Typhimurium that respond to norepinephrine, transposon mutagenesis and DNA microarray analysis were performed. Insertional mutations in the following genes decreased norepinephrine-enhanced growth: *degS*, *entE*, *entF*, *fes*, *gpmA*, *hfq*, STM3846. DNA microarray and real-time RT-PCR analyses revealed a decrease in the expression of several genes involved in iron acquisition and utilization during norepinephrine exposure, signifying the iron-limiting conditions of serum-SAPI minimal medium and the siderophore-like activity of norepinephrine. Unlike the wild-type parent strain, growth of neither a *fepA iroN cirA* mutant nor a *fepC* mutant, harboring deletional mutations in the outer and inner membrane transporters of enterochelin, respectively, was enhanced by norepinephrine. However, growth of the *fepC* and the *fepA iroN cirA* mutants could be rescued by an alternative siderophore, ferrioxamine E, further validating the role of norepinephrine in supplying the organism with iron via the catecholate-specific iron transport system. Contrary to previous reports using small animal models, the *fepA iroN cirA* mutant of *Salmonella* Typhimurium colonized the swine gastrointestinal tract, as did the *fepC* mutant.
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Keywords: *Salmonella enterica* serovar Typhimurium; Iron; Norepinephrine; Swine

1. Introduction

Bacteria possess the ability to sense, respond and adapt to environmental changes within and outside of the mammalian host. Adapting to these challenges is achieved via bacterial signaling mechanisms, such as two-component signal transduction systems, global transcriptional regulators and quorum sensing systems, which frequently results in changes in microbial gene expression. For example, an adaptation of quorum sensing bacteria to their in vivo environment is their ability of interspecies communication with the diverse, resident

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microbial community via the production of small hormone-like molecules called autoinducers (AIs) [1]. In addition, cellular communication may also occur between quorum sensing bacteria and their host. Sperandio and colleagues [2] have suggested that cross-talk between host hormones and the bacterial quorum sensing systems may occur. Thus, host hormones present in the gastrointestinal tract, such as norepinephrine, may notify enteric pathogens of their in vivo environment. In fact, in vitro investigations of the response of *Salmonella* and/or *Escherichia coli* to norepinephrine/epinephrine exposure revealed enhanced bacterial motility [2–4], induced cellular adherence [5,6], elevated virulence gene expression [7–9], and enhanced bacterial growth due to increased iron availability to the bacterial cell [10,11].

The acquisition of iron within the host is essential for bacterial survival, cellular metabolism and pathogenesis. Consequently, bacteria employ siderophores, iron specific scavengers, to sequester and transfer iron into the bacterial cell via specific transport proteins [12]. However, the host has developed innate immune responses that impede the iron acquisition activity of bacterial siderophores, for example lipocalin 2 sequesters enterochelin [13]. As a countermeasure to host lipocalin 2, products of the *iroA* gene cluster in *Salmonella* glucosylate enterobactin to produce salmochelin, thereby preventing lipocalin 2 binding and sequestration of enterobactin by the host [14–16]. Therefore, the competition for iron by both the host and the bacterial pathogen is notable since iron availability will in part determine the success of the pathogen within the host.

Although *Salmonella* (and other intracellular bacteria) require iron for survival and growth, excessive intracellular iron is detrimental to the organism, requiring tight regulation of iron acquisition and uptake [17]. In the presence of high levels of iron, the Fur protein serves as a negative regulator of genes involved in iron utilization [18]. In this study, numerous Fur-regulated, iron response genes exhibited a decrease in expression levels when *Salmonella* Typhimurium was grown in the presence of norepinephrine. The goal of this study was to investigate the role of iron-regulated genes in norepinephrine-enhanced growth and swine gastrointestinal disease caused by *Salmonella* Typhimurium, especially the outer membrane catecholate receptors FepA, IroN and CirA as well as the inner membrane enterochelin transporter FepC.

2. Materials and methods

2.1. Strains and media

The *Salmonella* Typhimurium strains used in the study are presented in Table 1 and were grown in Luria–Bertani (LB) broth or serum-SAPI minimal medium containing 2.77 mM dextrose, 6.25 mM ammonium nitrate, 1.84 mM monobasic potassium phosphate, 3.35 mM potassium chloride, and 1.01 mM magnesium sulfate, 30% porcine serum and 10 mM Hepes buffer [3]. Norepinephrine was used at concentrations of 50 μM and 2 mM. Norepinephrine is

photosensitive, highly oxidized and has a short half-life; therefore, two different concentrations were used in our studies for comparison to initial research in our laboratory and previously published data. Ferrioxamine E was used at a concentration of 5 ng/ml. Antibiotics were used at the concentrations of 100 μg/ml for ampicillin, 30 μg/ml for nalidixic acid, 30 μg/ml for chloramphenicol and 50 μg/ml for kanamycin. Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO). Cultures were grown and assays performed at 37 °C. Pre-conditioned serum-SAPI broth medium was prepared by diluting an overnight LB culture of BSX 8 20,000-fold in phosphate-buffered saline (PBS; BioWhittaker 17-512F). The cells were further diluted 100-fold in serum-SAPI broth medium containing 50 μM norepinephrine and incubated for 36–40 h at 37 °C with shaking. The culture was centrifuged to pellet the cells, and the supernatant was filter sterilized through a 0.2 μm filter and stored at 4 °C.

2.2. Transposon mutagenesis

Random transposon mutagenesis was performed in *Salmonella* Typhimurium UK1 by transforming the EZ-Tn5™ <R6Kγori/KAN-2>Tnp Transposome™ (Epicentre, Madison, WI) into 50 °C heat shocked (20 min), electrocompetent BSX 2. Transformed cells were plated on LB kanamycin to select for transposon mutants, and a transposon mutant bank was stocked in 100 96-well microtiter dishes and frozen at −80 °C. For screening of transposon mutants with decreased growth on serum-SAPI medium containing 50 μM norepinephrine, one-half of a microtiter dish (48 wells) was initially frogged to LB kanamycin and grown overnight. The LB kanamycin plate was then replicated to both serum-SAPI medium containing 50 μM norepinephrine and E glucose medium and incubated for 48 h at 37 °C. Colonies with reduced growth (based on a smaller colony size) on serum-SAPI medium containing 50 μM norepinephrine compared to E glucose medium were selected for further analysis by transduction of the EZ-Tn5™ transposon into BSX 8 (χ4232) using P22. The resulting strains are shown in Table 1.

2.3. Construction of mutants by recombineering

Recombineering was performed as described previously [3]. Briefly, PCR templates were used to produce linear knockout fragments using either oBBI 88/89 *cat* (encoding chloramphenicol acetyltransferase) or oBBI 92/93 *neo* (encoding the neomycin resistance gene) that were initially PCR amplified from pACYC184 (New England BioLabs, Beverly, MA) and pCR2 (Invitrogen, Carlsbad, CA), respectively. Both oBBI 88/89 *cat* and oBBI 92/93 *neo* have stop codons in all three reading frames on both ends of the DNA fragment. Additionally, FRT sites are located at the 5′ and 3′ ends of the PCR fragment to facilitate FLP catalyzed recombination resulting in deletion of the antibiotic resistance gene [19]. Each pair of knockout primers for a particular gene contains a ∼45 nucleotide sequence on the 5′ end that is homologous to the gene

Table 1
Strains and primers

Strain no.	Strain background	Genotype	Phenotype	Source
BSX 8	χ4232		Nal^R	Tom Stabel
BSX 7	TT22971 (LT2)	metA22 metE551 trpD2 ilv-452 leu pto (leaky) hsdLT6 hsdSA29 hsdB strA120/pKD46 araC bla oriR101 repA101ts lambda red (gam+ bet+ exo+)	Ap^R, 30 °C	John Roth via Max Wu
BSX 30	JF2043 (LT2)	*iroA*::MudJ *fur-1* zbf-5123::Tn10	Kn^R, Tet^R	John Foster
BBS 119	BSX 7 (LT2)	metA22 metE551 trpD2 ilv-452 leu pto (leaky) hsdLT6 hsdSA29 hsdB strA120	Ap^S, cured of pKD46	This study
BBS 120	BSX 7 (LT2)	metA22 metE551 trpD2 ilv-452 leu pto (leaky) hsdLT6 hsdSA29 hsdB strA120/pCP20	Ap^R, 30 °C	This study, pCP20 from Greg Philips
BBS 3	BSX 8 (χ4232)	*iroA*::MudJ	Nal^R Kn^R	This study, BSX 8 X HT BSX 30
BBS 24	BSX 8 (χ4232)	Δ*entA*	Nal^R	This study
BBS 46	BSX 8 (χ4232)	Δ*fepA*	Nal^R	This study
BBS 47	BSX 8 (χ4232)	Δ*iroN*	Nal^R	This study
BBS 80	BSX 8 (χ4232)	Δ*iroN* Δ*cirA*	Nal^R	This study
BBS 81	BSX 8 (χ4232)	Δ*fepA* Δ*iroN*	Nal^R	This study
BBS 83	BSX 8 (χ4232)	Δ*fepA* Δ*cirA*	Nal^R	This study
BBS 92	BSX 8 (χ4232)	Δ*cirA*	Nal^R	This study
BBS 93	BSX 8 (χ4232)	*entE*::EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 94	BSX 8 (χ4232)	*entE*::EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 95	BSX 8 (χ4232)	*hfq*::EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 96	BSX 8 (χ4232)	*entF*::EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 99	BSX 8 (χ4232)	Δ*iroBCDEN*	Nal^R	This study
BBS 108	BSX 8 (χ4232)	Δ*iroN* Δ*fepA* Δ*cirA*	Nal^R	This study
BBS 109	BSX 8 (χ4232)	Δ*iroC*	Nal^R	This study
BBS 110	BSX 8 (χ4232)	Δ*fepC*	Nal^R	This study
BBS 121	BSX 8 (χ4232)	Δ*iroE*	Nal^R	This study
BBS 122	BSX 8 (χ4232)	Δ*entS*	Nal^R	This study
BBS 134	BSX 8 (χ4232)	Δ*fepD*	Nal^R	This study
BBS 135	BSX 8 (χ4232)	Δ*fepG*	Nal^R	This study
BBS 136	BSX 8 (χ4232)	Δ*iroC* Δ*entS*	Nal^R	This study
BBS 140	BSX 8 (χ4232)	STM3846:EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 141	BSX 8 (χ4232)	*fes*:EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 142	BSX 8 (χ4232)	*degS*:EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 143	BSX 8 (χ4232)	*iroN*:EZ::Tn5 <R6Kγori/KAN-2>	Nal^R Kn^R	This study
BBS 158	BSX 8 (χ4232)	Δ*iroD*	Nal^R	This study
BBS 168	BSX 8 (χ4232)	Δfes	Nal^R	This study
BBS 295	BSX 8 (χ4232)	Δfes Δ*iroE*	Nal^R	This study
BBS 296	BSX 8 (χ4232)	*gpmA*::EZ::Tn5 <R6Kγori/KAN-2>	Kn^R	This study
BBS 298	BSX 8 (χ4232)	Δfes Δ*iroD*	Nal^R	This study
BBS 330	BSX 8 (χ4232)	Δ*iroN* Δ*fepA* Δ*cirA*/pBAD *fes fepA*	Nal^R Ap^R	This study
BBS 332	BSX 8 (χ4232)	Δ*fepC*/pBAD *fepDGC*	Nal^R Ap^R	This study
BBS 336	BSX 8 (χ4232)	Δ*fepD*/pBAD *fepDGC*	Nal^R Ap^R	This study
BBS 341	BSX 8 (χ4232)	Δfes Δ*iroD*/pBAD *fes fepA*	Nal^R Ap^R	This study
BBS 347	BSX 8 (χ4232)	Δ*fepG*/pBAD *fepDGC*	Nal^R Ap^R	This study

Gene	Primer sequences for recombineering (5′−3′)
entA	**gacgcctggtgggcgctgctttctcgcggggtagagtaatgaccg**catagagcagtgacgtagtcgc
	cgttcaattcatccagcgttaaatgccgtttccagatcatcgtcgatagctgaatgagtgacgtgc
fepA	**ggcttatgtggctaaagaaaagcaggatatacaatgaacaagaag**cgatagctgaatgagtgacgtgc
	gggaaggttgccatcagaaatgggtattaatgctcatataccacgcatagagcagtgacgtagtcgc
iroN	**gggcaacagcgctactttagacattatttagggaatgggtatgag**cgatagctgaatgagtgacgtgc
	gccagcggcgggcataatccatcaaaacgaggccgtcacaccggcgcatagagcagtgacgtagtcgc
cirA	**gtcgtatttacctcatggagaaatggaatgtttaggtttaaccct**cgatagctgaatgagtgacgtgc
	cgcggctgtcgccagtctctgtcagaaacggtaatccaccgccatcatagagcagtgacgtagtcgc
iroB	**gacgtggagagagaggatttctcatgcgtattctgtttgtcggt**gcatagagcagtgacgtagtcgc
	catattgtcattgcgctgccgcggttagccgtgttgcagcatggcgatagctgaatgagtgacgtgc
fes	**caaaattcataataaacacagggttataatgaaagaggcgctggc**cgatagctgaatgagtgacgtgc
	gaattccatactcaactcctgtcatggacatgtgcccggttaaagcatagagcagtgacgtagtcgc
fepC	**atgaccgaatccgtagcccgtttgcgcggcgaccagttaacgctg**cgatagctgaatgagtgacgtgc
	cgcccagcggtaccaccagcggcgtcccggcaaccggatcgtcgcatagagcagtgacgtagtcgc
iroC	**atgcccgcgactcattcccccatgcccgctcgtgcctggatagtt**gcatagagcagtgacgtagtcgc
	ctatcctatgacctctccttgcgtatcgcgtgtctggccgatgctcgatagctgaatgagtgacgtgc

Table 1 (*continued*)

Strain no.	Strain background	Genotype	Phenotype	Source
iroE	**ggaataacatccgatgtatggtcgccagtaccacaacaagcgcta**cgatagctgaatgagtgacgtgc			
	ctggcggcgggttaatggcagcgtggtttttggcttgccggttcgcatagagcagtgacgtagtcgc			
entS	**ctcgcattaaggcgttgtaatgaatcgacaatcctggctgctcaa**cgatagctgaatgagtgacgtgc			
	cagaccgatttggtcagtcggataaacgcccttcatcagccagcatagagcagtgacgtagtcgc			
fepD	**cagggaagttactatgtcatgctcgttttccgtgacgcgcgcgtt**cgatagctgaatgagtgacgtgc			
	gccgacgagaaagaaacatcacaggccgcctccgctcgatttacgcatagagcagtgacgtagtcgc			
fepG	**ccgtaaatcgagcggaggcggcctgtgatgtttctttctcgtcgg**cgatagctgaatgagtgacgtgc			
	cgcgcaaacgggctacggattcggtcatttttgcgggactcctgcatagagcagtgacgtagtcgc			
iroD	**gaccgaaatataaacgagatggaatacaaagaatgagactgagcc**gcatagagcagtgacgtagtcgc			
	ggcaggtgaagttttatcggggcagtagacgtaatccgtcaatcacgatagctgaatgagtgacgtgc			
	Primer sequences for real-time RT-PCR (5′–3′)			
cirA	gtcattacccagcaagatttac			
	cgaatactcacgcctttacg			
fepA	gaagattcattccctgaccttac			
	ggcagcggtcaccacgatgg			
iroN	gcagcaaagaaggtgacac			
	ccagctatcggcatcggttctg			
fepC	tctggctggatggcgaac			
	acggctgatgcggatacc			
iroC	catcattgtcatcgtagcatc			
	ggcaagtaacggcaggtgg			
sitB	ctgcgattaccgttgatcaagtc			
	ccggaaccatttacgcctacc			
fur	atgggtgaagaaatcggtctgg			
	gatggtcgtgatgatgctgttg			
degS	aatgaagtgacgccaaacgg			
	tcatcccgcattaccacgac			
entE	gctctgtttccccgctgatc			
	cactcctggatagcctgtag			
entF	ctatctcagcccgtcgtggtc			
	agcaaagtcgccagatgatgttc			
fes	cgctggcaacgggaagtg			
	cgtatcggggagtgtgtttcg			
hfq	gatttctactgttgtcccgtctcg			
	gtctcttcgctgtcctgttgc			
ryhB	gggaacccctacggagaacc			
	gcaaaagctggccaaaataatac			
STM3846	agaaactcagataagaagagaactattgc			
	cttaatgctactgccctttttatatgc			
gpmA	gcgtatgaagagcggtgagc			
	cggtcgggatgtttaactcaagg			
gyrB	gtcgaattcttatgactcctcc			
	cgtcgatagcgttatctacc			
	Primer sequences for complementation of mutations (5′–3′)			
fes fepA	ccatactcaactcctgtcatg			
	cgatatgcagcctgtgtccg			
fepDGC	ggagatatatcgccagcaatg			
	ccggctttgtcatcagtgtgg			

of interest (bold) and a universal sequence on the 3′ end to bind to the oBBI 88/89 *cat* or oBBI 92/93 *neo* template for PCR amplification (Table 1). Following PCR amplification and gel purification, the knockout fragment was transformed into arabinose-induced BSX 7 containing pKD46 [20]. Transformants were selected on LB containing the appropriate antibiotic and a single colony isolated. Phage was grown on the knockout mutant using P22 and transduced into BSX 8. The BSX 8 knockout mutant of each strain was transduced with P22 phage grown on BBS 120 containing pCP20 which encodes FLP recombinase [19]. Transductants were screened for loss of antibiotic resistance following induction of *flp* at 37 °C. The resulting *Salmonella* Typhimurium deletion mutants are shown in Table 1.

2.4. Construction of complementation plasmids

Primer sets (Table 1) were used to PCR amplify the *fes fepA* and *fepDGC* regions from the *Salmonella* Typhimurium BSX 8 genome. The appropriate sized PCR fragments were gel purified following electrophoresis on a 1% agarose gel. The DNA fragments were TOPO cloned into pBAD-TOPO (Invitrogen) and transformed into One Shot® TOP10 competent cells. Transformed cells were plated on LB Ap to select for *E. coli*

containing pBAD plasmids. The pBAD *fes fepA* and pBAD *fepDGC* plasmids were extracted from the TOP10 strains. Electrocompetent BBS 108 and BBS 298 were transformed with pBAD *fes fepA* while BBS 110, BBS 134 and BBS 135 were transformed with pBAD *fepDGC*.

2.5. Assessment of growth on serum-SAPI medium

Bacterial strains were grown overnight at 37 °C with shaking in 3 ml of LB containing appropriate antibiotics. Five-hundred microliter aliquots of overnight cultures were washed once with 500 µl of PBS. The cells were resuspended in 500 µl of PBS and diluted 500-fold in PBS. Serum-SAPI minimal medium plates containing 1.5% agar were spread with 100 µl of cells and allowed to dry for 30 min. A blank 6-mm paper disc (BBL 231039) was placed onto the center of each plate and one of the following was added directly to the disc: 20 µl of 62.5 mM norepinephrine (freshly prepared in serum-SAPI minimal medium), 20 µl of 6.25 µg/ml ferrioxamine E, or 25 µl of pre-conditioned serum-SAPI minimal medium. The diameters of growth zones around the discs were determined following incubation of the plates at 37 °C for 20 h. All strains were evaluated in at least three independent assays.

2.6. RNA isolation and purification

An overnight culture of BSX 8 in serum-SAPI minimal medium was diluted 1:100 in serum-SAPI minimal medium with or without 50 µM or 2 mM norepinephrine, grown to mid-log phase ($OD_{600} = 0.4$), and processed in RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). Total RNA was isolated and purified using the RNeasy Midi kit and RNase-free DNase set (Qiagen) and Turbo DNase-*free* kit (Ambion, Austin, TX). RNA integrity, quality and quantity were assessed using the Agilent Bioanalyser 2100 and RNA Nano 6000 Labchip kit (Agilent technologies, Palo Alto, CA).

2.7. DNA microarray analysis

RNA was labeled with aminoallyl dUTP via first strand cDNA synthesis followed by aminoallyl coupling to either Alexa 555 or 647 fluorescent molecules (Invitrogen) according to Protocol M007 from the Pathogen Functional Genomics Resource Center at The Institute for Genomic Research (TIGR; http://pfgrc.tigr.org/protocols.shtml). In dye swap hybridizations, labeled cDNA was hybridized to *Salmonella typhimurium/typhi* DNA microarrays (version 2) developed and manufactured by TIGR (http://pfgrc.tigr.org/desc.shtml) using Protocol M008. The ScanArray Express (PerkinElmer Life Sciences, Boston, MA) was used to scan the microarray slides and signal intensity measurements for each microarray spot were quantified using Scanarray Express 3.0 software. Background-corrected data were normalized using the Lowess function, and poorly measured spots were removed by filtering out those in which 50% or more of the pixels from both samples were within two standard deviations of the background.

The median intensity of the remaining spots was determined for each open reading frame, and ratios of the spot intensities for the experimental and control samples were calculated and log transformed for analysis on a minimum of three biological replicates.

2.8. Real-time RT-PCR

Real-time RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and the Chromo4 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA). Primer sequences are provided in Table 1. RT-PCR transcripts were amplified in duplicate from triplicate experiments with the following thermal cycling parameters: 50 °C for 30 min, 95 °C for 15 min, 35 cycles at 94 °C for 15 s, 60 °C for 60 s. At the 60 °C step, fluorescent data acquisition was performed. Following PCR cycling, disassociation curve analysis was performed from 55 °C to 90 °C with a plate reading at every 2 °C and a hold for 5 s at each temperature increment. Relative quantification of gene amplification by real-time RT-PCR was evaluated using the comparative C_T method as described by Livak and Schmittgen [21]. The gyrase subunit B gene, *gyrB*, was used as the endogenous normalization control. The ΔC_T values were calculated by determining the difference in threshold values for target and reference in each sample. Calculation of $\Delta\Delta C_T$ involved the subtraction of the no norepinephrine ΔC_T value from the ΔC_T value of the norepinephrine samples. Fold-differences in gene expression of the target gene are presented as $2^{-\Delta\Delta C}T$. Statistical analyses of the ΔC_T values were analyzed by SAS Analyst (Cary, NC) using the two sample *t*-test for the means. Results were considered significant when $P < 0.05$.

2.9. Swine study

Nine conventionally raised male and female piglets from *Salmonella* spp. fecal-negative sows were weaned at 12 days of age, shipped to the National Animal Disease Center, Ames, IA and raised in three groups of three in an isolation facility. Bacteriologic culture of rectal swabs was performed multiple times to confirm that all pigs were negative for *Salmonella* spp. At 10 weeks of age (day zero), all nine pigs received an intranasal inoculation of 1 ml of PBS containing 1.0×10^9 CFU of BSX 8, BBS 108 ($\Delta fepA$ $\Delta iroN$ $\Delta cirA$) or BBS 110 ($\Delta fepC$). Pig fecal samples were obtained on days 1–7 post-inoculation (p.i.) for quantitative and qualitative *Salmonella* culture analysis as previously described [3]. At 7 days p.i., the nine pigs were necropsied, and tissue samples of the gastrointestinal tract (cecum, ileal Peyer's Patch, ileocecal lymph nodes) as well as spleen and liver were placed on ice for quantitative and qualitative *Salmonella* culture [3]. Statistical analysis of the number of *Salmonella* present in the indicated tissues and daily fecal samples (cfu/g) was analyzed by SAS Analyst using the two sample *t*-test for the means. Procedures involving animals were lawful and approved by the USDA, ARS, NADC Animal Care and Use Committee.

3. Results

3.1. Transposon mutagenesis identifies iron response genes involved in norepinephrine-enhanced growth of Salmonella Typhimurium

In serum-SAPI minimal medium, the growth of *Salmonella* Typhimurium is dramatically enhanced in the presence of norepinephrine or pre-conditioned medium containing autoinducer [22]. Transposon mutagenesis was performed to identify *Salmonella* Typhimurium genes required for norepinephrine-enhanced growth on serum-SAPI minimal medium containing 50 μM norepinephrine. Transposon mutants that grew slowly were selected for further characterization based on their smaller colony size. DNA sequence analysis of the transposon junction region for the *Salmonella* Typhimurium mutants that exhibited decreased growth in the presence of norepinephrine identified the following genes: *degS*, *entE*, *entF*, *fes*, *gpmA*, *hfq*, and STM3846. The zone diameters of growth for each of the mutants in the presence of norepinephrine, pre-conditioned (spent) medium, or ferrioxamine E are presented in Table 2. To determine if these genes are transcriptionally altered during norepinephrine exposure, real-time RT-PCR was performed using total RNA from the parent wild-type strain grown in serum-SAPI minimal medium with and without 50 μM norepinephrine (Fig. 1A). Except for the *degS* gene, each of the genes assayed had significantly lower levels of transcription in the presence of norepinephrine compared to its absence. The involvement of several Fur-regulated, iron response genes (*entE*, *entF*, *fes* and *gpmA*) in NE-enhanced growth and the altered expression of the *hfq* gene, encoding an RNA chaperone required for stability of several small antisense regulatory RNAs including RyhB [23] suggested that the *ryhB* gene may also be differentially expressed in the presence versus absence of norepinephrine: a 13-fold decrease in transcription was observed for the small regulatory RNA during norepinephrine exposure.
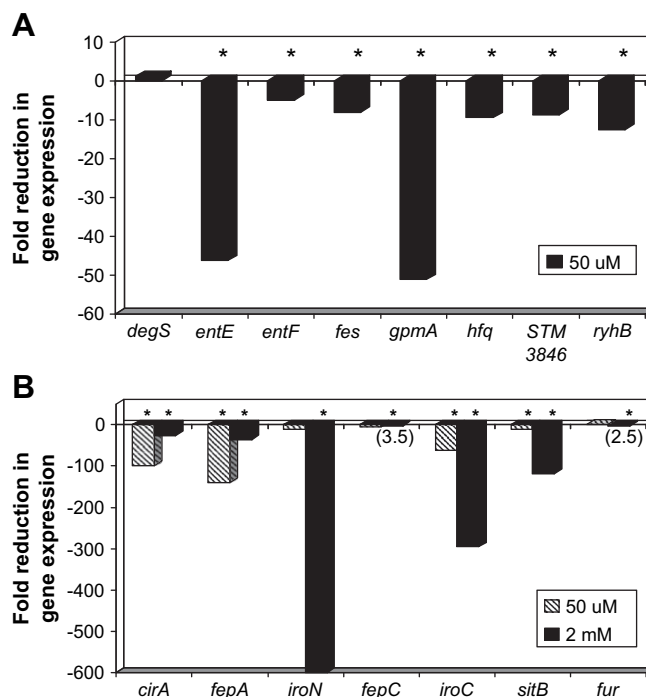

Fig. 1. Real-time RT-PCR analysis of wild-type *Salmonella* Typhimurium in the presence and absence of norepinephrine. BSX 8 was grown in serum-SAPI minimal medium with or without 50 μM or 2 mM norepinephrine to an $OD_{600} = 0.4$ and processed for RNA isolation. (A) Transcriptional analysis of genes identified by transposon mutagenesis as required for norepinephrine-enhanced growth. (B) Validation and quantitation of transcriptional repression of iron-response genes during growth with norepinephrine. Results were considered significant (*) when $P < 0.05$.

3.2. DNA microarray analysis confirms the iron-limiting environment of serum-SAPI minimal medium and the siderophore-like activity of norepinephrine

Global investigation of the transcriptional profile of *Salmonella* Typhimurium to norepinephrine exposure by DNA microarray analysis revealed 36 iron response genes with ≥1.5

Table 2
Decreased growth of *Salmonella* Typhimurium transposon mutants on serum-SAPI minimal medium

Strain	Genotype	STM no.[a]	Insertion site duplication/no. of bp[b]	Description	NE[c]	PC[d]	FE[e]
BSX 8	Wild-type				3.0	1.9	3.3
BBS 93	entE	STM0596	1455–1465/1611	2,3-Dihydroxybenzoate-AMP ligase	0.6	1.2	3.4
BBS 94	entE	STM0596	388–396/1611	2,3-Dihydroxybenzoate-AMP ligase	0.6	1.1	3.4
BBS 95	hfq	STM4361	187–196/309	Host factor I	2.6	1.6	3.4
BBS 96	entF	STM0588	3757–3765/3885	Enterobactin synthetase component F	0.6	1.2	3.4
BBS 140	STM3846	STM3846	619–622/870	Putative reverse transcriptase	2.9	2.1	3.3
BBS 141	fes	STM0586	195–203/1215	Enterochelin esterase	2.9	1.9	3.3
BBS 142	degS	STM3349	813–822/1071	Periplasmic serine endoprotease	2.2	1.2	2.6
BBS 296	gpmA	STM0772	444–453/753	Phosphoglyceromutase	0.6	0.6	0.6

[a] STM prefix denotes the gene number on the chromosome as assigned by the *Salmonella enterica* serovar Typhimurium LT2 complete genome sequencing project.

[b] Location of the transposon insertion site duplication within the gene/number of base pairs within the gene.

[c] Growth zone diameter (cm) around a disc with 20 μl of 62.5 mM norepinephrine on serum-SAPI agar medium.

[d] Growth zone diameter (cm) around a disc with 25 μl of pre-conditioned serum-SAPI medium (from BSX 8) on serum-SAPI agar medium.

[e] Growth zone diameter (cm) around a disc with 20 μl of 6.25 μg/ml ferrioxamine E on serum-SAPI agar medium.

fold decreased expression (out of a total 88 genes) in the presence of 2 mM norepinephrine compared to cultures grown without norepinephrine (Fig. 2 and Supplementary Table 1). Transcriptional analysis employing an additional DNA microarray platform [24] also identified lower expression of iron response genes during norepinephrine exposure (data not shown). Therefore, elevated expression of the iron-regulated genes in serum-SAPI minimal medium indicates an iron limited environment that can be compensated with the addition of norepinephrine. From the DNA microarray results, several genes involved in iron acquisition and utilization were selected for real-time RT-PCR analysis. Fig. 1B illustrates the decreased transcriptional levels of *cirA*, *fepA*, *iroN*, *fepC*, *iroC* and *sitB* in the presence of 50 μM and/or 2 mM norepinephrine. Since many of the genes are regulated by the global transcriptional iron regulator Fur, real-time RT-PCR was also performed for the *fur* gene; a significant, 2.5-fold reduction in gene expression was observed in the wild-type strain grown with 2 mM norepinephrine.

3.3. Enterochelin transport proteins required for norepinephrine-enhanced growth of Salmonella

Transcription of the genes encoding the catecholate siderophore receptors, FepA, IroN, and CirA was decreased in the presence of norepinephrine. Single, double and triple mutants were constructed for the catecholate receptors as well as other enterochelin/salmochelin synthesis and transport genes. Unlike the parent strain, bacterial growth was not enhanced in the *fepA iroN cirA* mutant with the addition of norepinephrine (Table 3), phenotypically confirming the involvement of the three outer membrane proteins in norepinephrine-facilitated iron uptake, as suggested by Williams et al. [25]. Furthermore, mutants of the *fepDGC* operon, encoding components of the ferric enterobactin transport system complex located at the inner membrane, also displayed a dramatic decrease in norepinephrine-enhanced growth. To further confirm the role of norepinephrine in iron acquisition

via the enterochelin transport system, growth on serum-SAPI minimal medium containing ferrioxamine E, a siderophore that utilizes a different outer membrane receptor (FoxA) and inner membrane transport system (FhuBCD) [26] was performed for several of the constructed mutants. As shown in Table 3, providing the enterochelin transport deficient *fepA iroN cirA* triple mutant or mutants of the *fepDGC* operon with an alternative siderophore resulted in bacterial growth, indicating that NE supplies *Salmonella* with iron in a siderophore-like manner and requires the catecholate transporters.

3.4. Overlapping enzymes involved in enterochelin acquisition

Three enzymes hydrolyze enterochelin and/or salmochelin, Fes, IroD and IroE. Our results indicate that individual mutations in any of these genes have only minor decreases in growth on serum-SAPI minimal medium in the presence of norepinephrine. However, a *fes iroD* double mutant displayed a dramatic decrease in norepinephrine-enhanced growth (Table 3), suggesting redundancy in the enzymes that are required for norepinephrine-enhanced growth.

3.5. Catecholate transport mutants of Salmonella Typhimurium are not attenuated for swine colonization

Rabsch et al. [27] and Williams et al. [25] report an attenuated phenotype for a *fepA iroN cir* mutant in the mouse model for systemic disease of *Salmonella* Typhimurium. To test the virulence of the triple mutant as well as a *fepC* mutant in a enterocolitis disease model of swine, BBS 108 (*fepA iroN cirA*), BBS 110 (*fepC*) and BSX 8 (wild-type parent strain) were intranasally inoculated into 10-week-old pigs (*n* = 3 for each strain). No significant difference in colonization of the three strains was observed in the Peyer's Patch region of the ileum, ileocecal lymph nodes or cecum at 7 days p.i. (Fig. 3), nor was a difference observed in daily fecal shedding
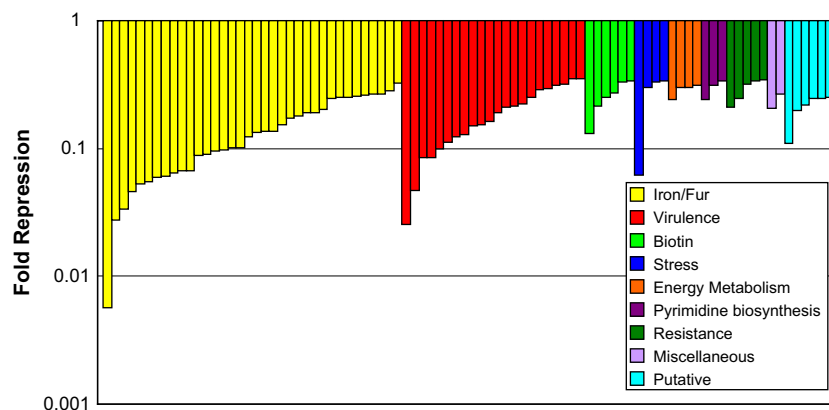

Fig. 2. Transcriptional repression of *Salmonella* Typhimurium genes during norepinephrine exposure. The histogram represents various classes of *Salmonella* Typhimurium genes identified by DNA microarray analysis as transcriptionally repressed in the presence of norepinephrine (log2 less than-1.5). Supplementary Table 1 provides specific information of gene identify, product function, log2 repression and fold change in expression of the genes represented in the histogram.

Table 3
Decreased growth of *Salmonella* Typhimurium mutants on serum-SAPI minimal medium

Strain	Genotype	STM no.[a]	Description	NE[b]	PC[c]	FE[d]
BSX 8	Wild-type			3.0	1.9	3.3
BBS 3	*iroA*::MudJ	STM2774	MudJ insertion in *iroC*	2.3	1.1	2.2
BBS 10	*ygiY*::cat	STM3178	Quorum sensor kinase, QseC	2.9	1.7	
BBS 24	Δ*entA*	STM0598	2,3-Dihydroxybenzoate-2,3-dehydrogenase	0.6	1.1	1.8
BBS 46	Δ*fepA*	STM0585	Outer membrane ferric enterobactin receptor precursor	2.8	1.8	
BBS 47	Δ*iroN*	STM2777	TonB-dependent outer membrane siderophore receptor	2.8	1.4	
BBS 92	Δ*cirA*	STM2199	Iron-catecholate outer membrane transporter	2.8	2.0	
BBS 81	Δ*fepA* Δ*iroN*			2.8	0.9	
BBS 80	Δ*iroN* Δ*cirA*			2.4	1.4	
BBS 83	Δ*fepA* Δ*cirA*			2.7	1.8	
BBS 108	Δ*fepA* Δ*iroN* Δ*cirA*			0.6	0.6	3.2
BBS 330	Δ*fepA* Δ*iroN* Δ*cirA*/pfes fepA			2.7	1.4	2.8
BBS 99	Δ*iroBCDEN*			3.0	1.7	
BBS 109	Δ*iroC*	STM2774	Putative ABC transporter protein	2.5	1.6	
BBS 110	Δ*fepC*	STM0590	Iron-enterobactin transporter ATP binding protein	0.6	0.6	3.1
BBS 332	Δ*fepC*/pfepDGC			2.8	1.6	3.0
BBS 134	Δ*fepD*	STM0592	Iron-enterobactin membrane transport protein	0.6	0.6	3.2
BBS 336	Δ*fepD*/pfepDGC			3.1	1.7	3.0
BBS 135	Δ*fepG*	STM0591	Iron-enterobactin transporter permease	0.6	0.6	3.3
BBS 347	Δ*fepG*/pfepDGC			2.5	1.3	2.7
BBS 122	Δ*entS*	STM0593	Enterobactin transporter; aka *ybdA*	2.5	1.5	
BBS 136	Δ*iroC* Δ*entS*			2.8	1.7	
BBS 121	Δ*iroE*	STM2776	Salmochelin/enterochelin siderophore esterase	2.8	1.6	3.2
BBS 158	Δ*iroD*	STM2775	Salmochelin/enterochelin siderophore esterase	2.6	1.5	2.7
BBS 168	Δ*fes*	STM0586	Ferric enterochelin esterase	2.8	1.9	3.2
BBS 295	Δ*fes* Δ*iroE*			3.0	1.7	3.3
BBS 298	Δ*fes* Δ*iroD*			0.6	0.6	2.8
BBS 341	Δ*fes* Δ*iroD*/pfes fepA			2.8	1.7	2.9

[a] The STM prefixes indicate the gene numbers on the chromosome as assigned by the *Salmonella enterica* serovar Typhimurium LT2 complete genome sequencing project.

[b] Growth zone diameter (cm) around a disc with 20 μl of 62.5 mM norepinephrine on serum-SAPI agar medium.

[c] Growth zone diameter (cm) around a disc with 25 μl of pre-conditioned serum-SAPI medium (from BSX 8) on serum-SAPI agar medium.

[d] Growth zone diameter (cm) around a disc with 20 μl of 6.25 μg/ml ferrioxamine E on serum-SAPI agar medium.

of *Salmonella* from the pigs during the 7-day experiment (data not shown). As usually seen in our swine model of infection for *Salmonella* Typhimurium, *Salmonella* was not detected in the spleen or liver at 7 days p.i.
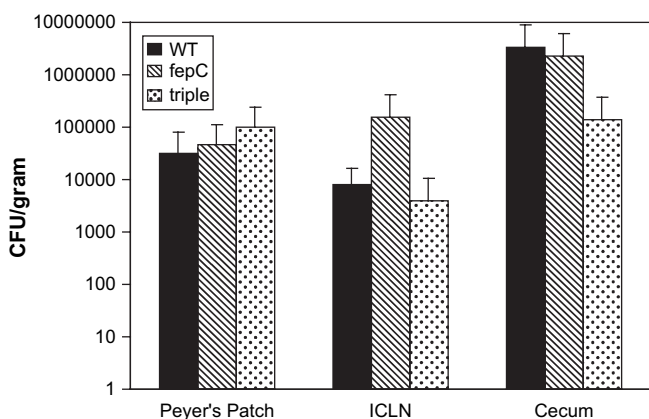

Fig. 3. Neither the *fepA iroN cirA* mutant nor the *fepC* mutant are attenuated for virulence in the swine gastrointestinal model of *Salmonella* Typhimurium infection. Ten-week-old pigs were intranasally inoculated with 1.0×10^9 CFU of either BSX 8 (WT), BBS 110 (*fepC*) or BBS 108 (*fepA iroN cirA*) ($n = 3$ for each strain). At 7 days p.i., quantitative and qualitative bacterial culturing was performed for *Salmonella* in the following tissues: Peyer's Patch region of the ileum (PP), ileocecal lymph nodes (ICLN) and cecum.

4. Discussion

Iron modulation in the host during microbial infection can influence the outcome of disease [28−30]. For example, individuals with iron-deficient macrophages due to hereditary hemochromatosis gene mutations appear to have elevated resistance to microorganisms that replicate in macrophages (such as *Salmonella*, *Legionella*, *Mycobacteria*, *Chlamydia*, *Yersinia*) [31]. Selection for this inherited mutation may have occurred during the European plagues due to the C282Y mutation conferring resistance to *Yersinia* infection [32]. Moreover, several pathogenic enteric bacteria, including *Salmonella*, convert enterochelin to salmochelin to circumvent sequestration of enterochelin by host lipocalin 2 [15], emphasizing the importance of iron acquisition by microorganisms for their in vivo survival, proliferation and virulence.

Investigations of enteric pathogens during exposure to the stress hormone norepinephrine revealed bacterial growth enhancement and motility induction via quorum sensing signaling [3,9,10,33]. Enhanced proliferation has been shown to involve the siderophore-like activity of norepinephrine supplying iron to the bacterial cell. Our transposon mutagenesis, DNA microarray and real-time RT-PCR analyses provide a comprehensive examination of *Salmonella* Typhimurium genes involved in norepinephrine-enhanced growth, illustrating a higher expression

level for many iron-regulated genes during growth in iron-limiting, serum-SAPI minimal medium and a decrease in the expression of these genes in the presence of norepinephrine. It may seem counterintuitive that the expression of iron transport and utilization genes would be down-regulated when norepinephrine is present and providing *Salmonella* with iron. Keep in mind, excess iron can be toxic, requiring tight regulation of the iron response genes. When iron is available, Fur binds iron, and the iron-Fur complex binds to a DNA sequence referred to as the Fur box in the promoter region of iron regulated genes, thereby repressing transcription. Our transcriptional assays revealed lower expression levels of many Fur-regulated iron response genes, apparently due to norepinephrine furnishing iron to *Salmonella*. For instance, genes involved in enterochelin synthesis (*entE*, *entF*), transport (*iroN*, *fepC*, *cirA*, *fepC*, *iroC*) and breakdown (*fes*) were identified in either the mutagenesis study (to identify genes required for norepinephrine-enhanced growth) or DNA microarray analysis (to compare gene transcription in an iron-limiting medium with and without norepinephrine). Other investigators have described a requirement for the catecholate siderophore receptors FepA, IroN, and CirA in norepinephrine-facilitated iron uptake [25,27]. In our study, we demonstrate a requirement of the ferric enterobactin transport complex encoded by *fepDGC* for norepinephrine-enhanced growth of *Salmonella* Typhimurium. Therefore, norepinephrine-enhanced growth requires enterochelin synthesis (*entE*, *entF* and *entA*), siderophore transport across the outer (*iroN*, *fepA*, *cirA*) and inner (*fepDGC*) membranes and siderophore hydrolysis in the cytoplasm (*fes*, *iroD*).

From our real-time RT-PCR data, a trend was detected for several iron-regulated genes (*iroN*, *iroC* and *sitB*) to have greater repression in the presence of an increased concentration of norepinephrine (2 mM vs. 50 µM, Fig. 1B). However, a surprising but repeatable result was observed with both *cirA* and *fepA* having greater repression in the presence of 50 µM norepinephrine compared to 2 mM norepinephrine. Although the authors are unclear of the significance of these results, consider that these experiments are a snapshot in time and not a progression of gene expression changes over time. Moreover, differential control of the catecholate receptors may occur since the IroN receptor can transport salmochelin, enterochelin and breakdown products of both of these siderophores, whereas CirA only transports the siderophore breakdown products and FepA transports enterochelin and its breakdown products [16]. Greater transcriptional repression of the *iroA* locus (including *iroN*) is supported by our microarray data.

Another obvious group of genes that are down-regulated in *Salmonella* Typhimurium during norepinephrine exposure are genes that function in the Type III secretion system, especially those encoded by *Salmonella* pathogenicity island 2 (SPI2). The role of SPI2 in *Salmonella* virulence has traditionally been associated with intracellular survival, systemic disease and persistent infection [34], however recent investigations have revealed a need for SPI2 in intestinal pathogenesis [35]. Similar to the decrease in SPI2 transcription observed in the presence of norepinephrine in our study (an iron-rich

environment), Zaharik et al. [36] demonstrated that chelation of iron up-regulates the expression of *Salmonella* Typhimurium SPI2 virulence genes. Thus, an iron deplete environment (as encountered during systemic infection) triggers the expression of the SPI2 genes required for the establishment of systemic disease.

As our attempts to mimic in vivo conditions with serum-SAPI minimal medium identified many enterochelin synthesis and transport genes involved in norepinephrine-enhanced growth, mutations were constructed in the catecholate siderophore transporters for colonization studies in the swine enterocolitis model for *Salmonella* Typhimurium infection. Contrary to previous reports in the mouse model, the *fepA iroN cirA* mutant was not attenuated for colonization, and neither was the *fepC* mutant. Similar to most human infections, *Salmonella* Typhimurium in swine usually causes only enterocolitis. One could propose that localization of *Salmonella* Typhimurium to the porcine gastrointestinal tract may provide an environment for hijacking of siderophores produced by the swine flora; in so doing, the enterochelin transport mutants could survive and colonize the intestinal tract of the pig. An indication of the ability for *Salmonella* to prevail over the loss of the enterochelin transporters for iron acquisition was evident with the growth of the *fepA iroN cirA* and *fepC* mutants on medium containing a substitute siderophore, ferrioxamine E. On the other hand, systemic infections, as observed in the mouse model, may represent an iron-deplete environment without competing flora serving as alternative siderophore providers. Thus, under systemic conditions, a *fepA iroN cirA* mutant may present a more dramatic decrease in colonization and disease, explaining the differences observed in the virulence status of the catecholate transport mutants in the different animal studies. This investigation raises a noteworthy issue: genes of *Salmonella* Typhimurium identified in the mouse model (a systemic infection) to be avirulent and a potential vaccine candidate may not be attenuated in larger mammals (such as pigs and humans) during gastrointestinal infections.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micinf.2008.04.011.

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